Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model

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CHAPTER 2

Validation of a novel type 1 diabetic model for in-stent restenosis after rat abdominal aortic stenting.

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ABSTRACT

Aim: Diabetic animal models are useful for studying the mechanism of increased in-stent restenosis in diabetic populations. We aimed to establish a novel type 1 diabetic model for in-stent restenosis.

Methods: Thymectomy was performed on 6 young BB-DP(Bio-Breeding Diabetes-Prone)to prevent the development of diabetes and create a non-diabetic group, 6 other age-macthed BB-DP from the same breeding population were allowed to develop diabetes. At the age of nine months all 12 animals were implanted with a stent in the abdominal aortic and after 28 days stented abdominal aortas were harvested, embedded in plastic, cut, stained and analyzed.

Results: Neointimal area was increased in the non-thymectomized BB-DP rats compared with the thymectomized BB-DP rats. Furthermore there was significant proteinuria, and polyuria in diabetic non-thymectomized BB-DP .

Conclusions: These results validate this novel type 1 diabetic rat abdominal aortic stenting model for studying the mechanism of increased in-stent restenosis in diabetic populations and more specific in the type 1 diabetes population.

INTRODUCTION

Diabetes is a risk factor for in-stent restenosis even with the use of drug-eluting stents[1]. Increased neointimal formation in diabetic patients is the cause of increased clinical in-stent restenosis in diabetic patients[2]. Diabetic animal models are useful for studying the mechanism of increased in-stent restenosis in diabetic populations [3]. Although a type 2 diabetic restenosis model has been established[4], a reliable type 1 diabetic restenosis model has not been described yet. Diabetes type 1 patients represent only 5-10% of all diabetes patients, however these patients often have severe coronary artery disease at a young age[5]. A type 1 diabetic restenosis swine model using streptozotocin to induce diabetes was developed by Carter et al[6]. However the high reported mortality (45%) of diabetic animals in this study may limit the practicality of this model[6]. We present in this study a novel and reliable type 1 diabetic model for in-stent restenosis after rat abdominal aortic stenting. Hyperglycemia, proteinuria, polyuria, weight and neointimal area were measured to validate this type 1 diabetic model for in-stent restenosis.

METHODS

Animals

All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Specific pathogen-free, Bio-Breeding Diabetes-Prone were bred at the Central Animal Facility of the University Medical Center Groningen. Original breeding stocks were obtained from BRM Inc.(Worchester, MA,USA). If thymectomized BB-DP do not develop diabetes[7]. Rats were kept under clean conventional conditions and were fed standard rat chow and acidified water ad libitum.

Animal Protocol

Diabetes measurements

Thymectomy was performed on 6 BB-DP to prevent the development of diabetes and create a non-diabetic control group. Thymectomy was performed on young rats (age 21 days) as described in detail by Visser et al[7]. In our breeding colony of BB-DP rats 90% of rats have diabetes at an age of 70 days. 6 age-matched non-thymectomized BB-DP were selected from our breeding population to form the diabetic population.

Blood-glucose levels were measured in blood from the tail vein of non-diabetic thymectomized BB-DP(n=6) and diabetic non-thymectomized BB-DP(n=6) with an Accu-Chek Sensor Comfort glucose strips (Roche Diagnostics Nederland B.V., Almere The Netherlands). Diabetic non-thymectomized BB-DP animals were also checked for weight loss three times a week. If significant weight loss occurred combined with glucose exceeding 20 mM, the diabetic animals were treated with an insulin pellet (Lin-Plant; LinShin Canda Inc, Toronto, ON, Canada) to prevent severe diabetic dysregulation which is usually followed by death. Half an insulin pellet was inserted through a small incision in the scruff of the neck with a heavy-gauge needle. The insulin pellet released insulin at a steady state, however the insulin released by half the pellet was deliberately dosed too low too ensure hyperglycemic episodes in the diabetic animals. Urine production was determined before stent-implantation and at the end of the study by placing the rats in individual, urine-collecting metabolic cages for 24 hours. Total urine protein excretion was determined with the U/CSF protein assay (Roche, Woerden, The Netherlands).

Stent implantation
At the age of nine months animals were anesthetized with O2, N2O, and isoflurane 2% (Abbott International Ltd). Premounted, 2.5 x 9 mm BeStent^{tm} 2 (Medtronic-Bakken Research, Maastricht, The Netherlands) n=6, bare metal stents were implanted in the abdominal aorta as described previously[8]. Both non-diabetic thymectomized BB-DP(n=6) and diabetic non-thymectomized BB-DP(n=6) received a stent. After 28 days animals were anesthetized with O2, N2O, and isoflurane 2%, systemically heparinized with 500 IU i.v.(Leo Pharma, Breda, The Netherlands). Abdominal aortas were harvested and fixed in 4% formalin (Klinipath, Duiven, The Netherlands), buffered at pH 6.5.

Histology
Histomorphometrical analysis was performed on Lawson (elastin) stained sections by measurements of the proximal, middle, and distal parts of each stent. The neointimal area, media area and lumen area were measured or calculated as described previously[8]. In short, the areas within the external elastic lamina (EEL), internal elastic lamina (IEL) and lumen were measured by using digital morphometry by means of an Olympus BX-50F4 microscope, an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). The lumen area was substracted from the IEL area to give the neointimal area.

Statistical analysis
Data are expressed as mean ±SEM. Differences between groups were determined by an independent samples *t* test. All *P*-values were two-tailed, and a *P*-value of <0.05 was considered statistically significant. Analyses were performed using SPSS software (SPSS version 12.0, Chicago, IL, USA).

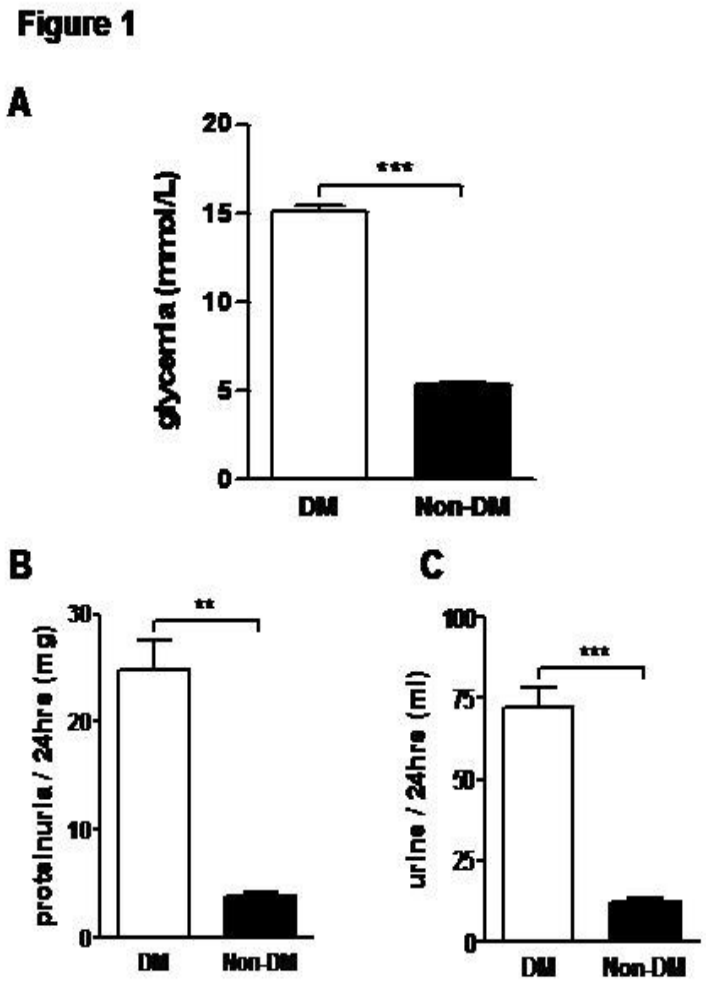

Figure 1: Hyperglycemia, proteinuria and polyuria is present in non-thymectomized BB-DP rats but not in thymectomized BB-DP rats.

RESULTS

Diabetes parameters

The non-thymectomized BB-DP rats developed diabetes at an median age of 82 days. Thymectomized BB-DP control rats did not develop diabetes. Mean blood glucose level after diabetes onset in diabetic non-thymectomized BB-DP rats was 15.1±0.3mmol/L versus 5.3±0.1mmol/L in non-diabetic thymectomized BB-DP control rats (Figure 1). Furthermore there was significant proteinuria, and polyuria in diabetic non-thymectomized BB-DP (Figure 1).

Figure 2

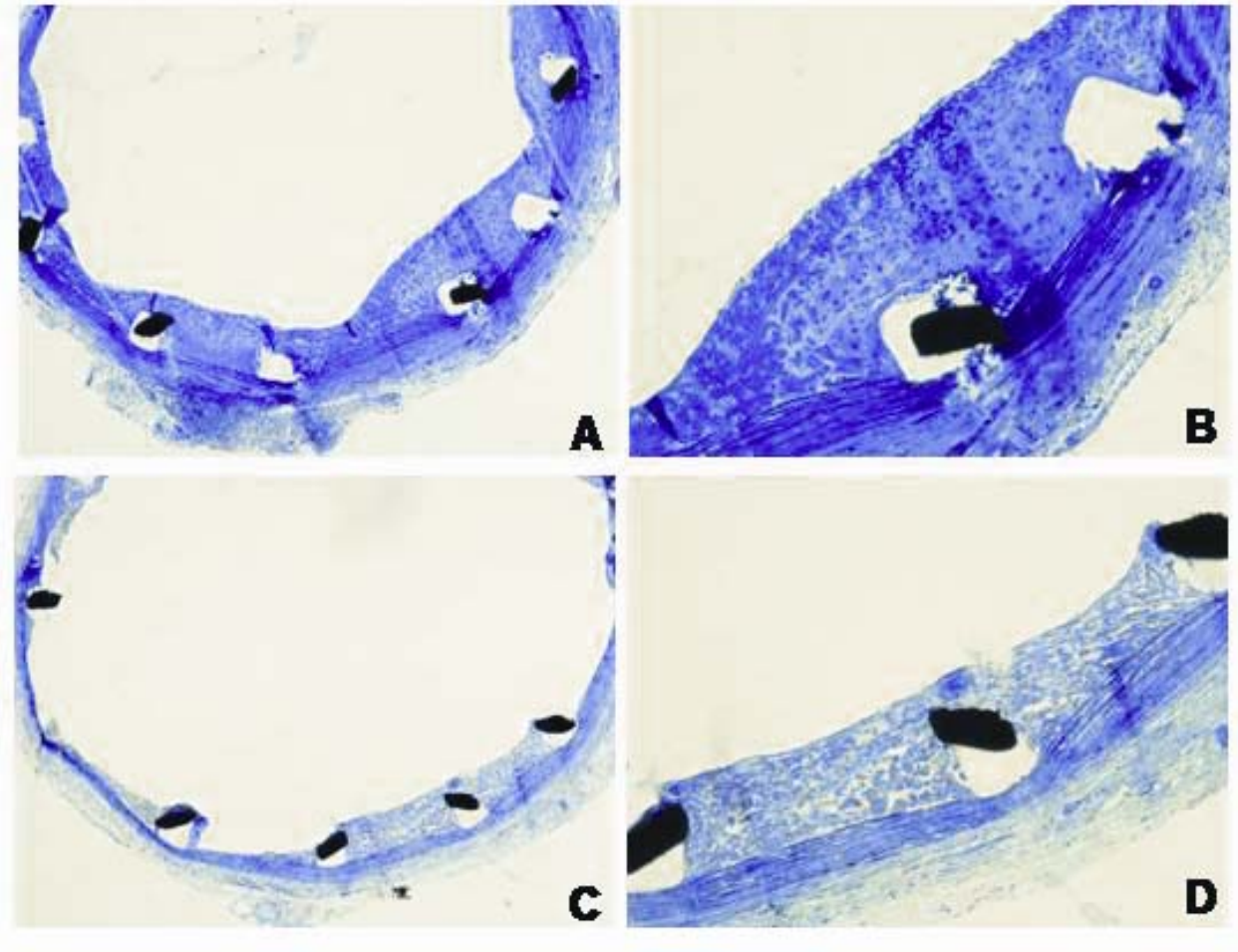

Figure 2: Photomicrographs of stented abdominal aortas showing the neointima, internal elastic lamina , external elastic lamina, stent struts and A and B(diabetes),C and D (normoglycemic, x40 and x400).

Neointimal formation

In diabetic non-thymectomized BB-DP neointimal area was significantly increased 0.69±0.02 mm^2 compared to 0.53±0.06 mm^2 in non-diabetic thymectomized BB-DP control rats (Figure 2 and 3).

Figure 3

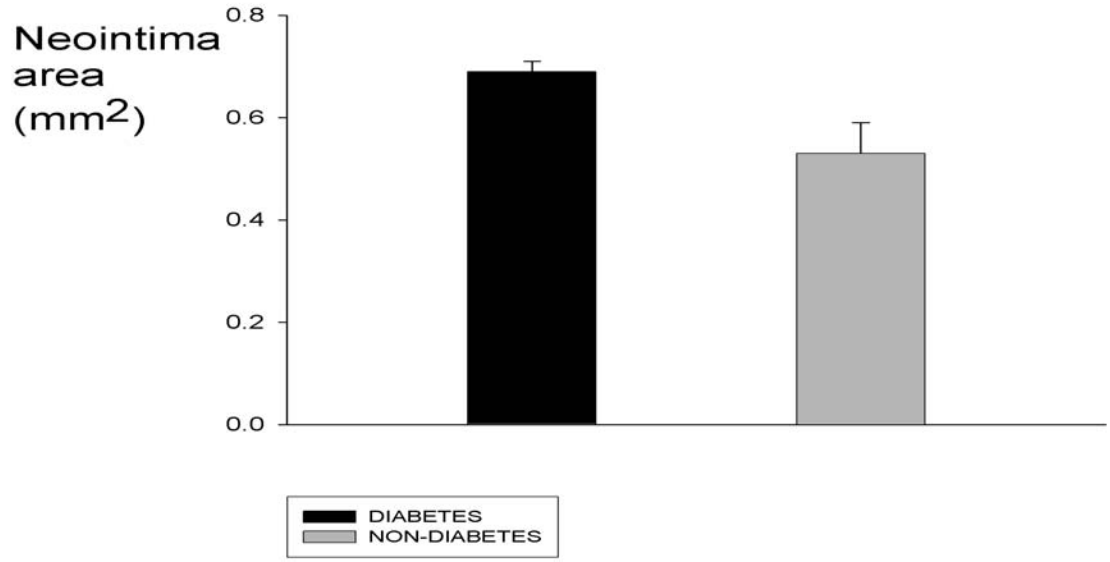

Figure 3: Neointimal area is increased in diabetic animals.

DISCUSSION

In this study we demonstrated increased neointimal formation in diabetic BB-DP rats compared with BB-DP normoglycemic rats who are genetically similar but do not develop diabetes due to a thymectomy at young age. The diabetic BB-DP had proteinuria, polyuria and raised glucose values of diabetes whereas the thymectomized BB-DP had not. The increase in neointimal formation in the diabetic animals corresponds with results in humans. Because of the genetic similarity of the diabetic and normoglycemic rats in this model the mechanisms responsible for increased in-stent restenosis in diabetic populations can more easily be identified in future studies. Furthermore the feasibility of treating these rats with insulin pellets as shown in this study will enable studies on the relation between glucose levels and in-stent restenosis. Limitation of this study is that the effect of thymectomy on in-stent restenosis was not studied. Before the adult age of ten weeks in rats the thymus is needed for T-cell development. Therefore the juvenile rats of three weeks in our study have an impairment in T-cell mediated immune response. This impairment in T-cell mediated immune response prevents the development of diabetes in this model. However it could also affect the development of in-stent restenosis. One study found activation of T-cells after percutaneous transluminal coronary angioplasty[9]. However treatment with cyclosporine a powerful specific inhibitor of the T-cell mediated immune response did not reduce restenosis in a cholesterol-clamped rabbit animal model[10]. So the T-cell mediated immune response does not attribute significantly to in-stent restenosis. Therefore it is not likely that thymectomy affected in-stent restenosis in this animal model. These results validate this novel type 1 diabetic rat abdominal aortic stenting model for studying the mechanism of increased in-stent restenosis in diabetic populations and more specific in the type 1 diabetes population.

Acknowledgments We thank Annemiek Smit-van Oosten and Anthony van Dijk for their excellent biotechnical assistance.

References

1. Scheen AJ, Warzee F. Diabetes is still a risk factor for restenosis after drug-eluting stent in coronary arteries. *Diabetes Care* 2004;27:1840-1841.

2. Kornowski R, Mintz GS, Kent KM, Pichard AD, Satler LF, Bucher TA, Hong MK, Popma JJ, Leon MB. Increased restenosis in diabetes mellitus after coronary interventions is due to exaggerated intimal hyperplasia. A serial intravascular ultrasound study. *Circulation* 1997;95:1366-1369.

3. Takeda R, Suzuki E, Satonaka H, Oba S, Nishimatsu H, Omata M, Fujita T, Nagai R, Hirata Y. Blockade of endogenous cytokines mitigates neointimal formation in obese Zucker rats. *Circulation* 2005;111:1398-1406.

4. Shelton J, Wang D, Gupta H, Wyss JM, Oparil S, White CR. The neointimal response to endovascular injury is increased in obese Zucker rats. *Diabetes Obes Metab* 2003;5:415-423.

5. Dickinson S, Rogers T, Kasiske B, Bertog S, Tadros G, Malik J, Wilson R, Panetta C. Coronary artery disease in young women and men with long-standing insulin-dependent diabetes. *Angiology* 2008;59:9-15.

6. Carter AJ, Bailey L, Devries J, Hubbard B. The effects of uncontrolled hyperglycemia on thrombosis and formation of neointima after coronary stent placement in a novel diabetic porcine model of restenosis. *Coron Artery Dis* 2000;11:473-479.

7. Visser J, Klatter F, Hillebrands JL, Jansen A, Vijfschaft L, Rozing J. Thymectomy should be the first choice in the protection of diabetes-prone BB rats for breeding purposes. *Lab Anim* 2004;38:371-375.

8. Langeveld B, Roks AJ, Tio RA, van Boven AJ, van der Want JJ, Henning RH, van Beusekom HM, van der Giessen WJ, Zijlstra F, van Gilst WH. Rat abdominal aorta stenting: a new and reliable small animal model for in-stent restenosis. *J Vasc Res* 2004;41:377-386.

9. Osada M, Takeda S, Ogawa R, Komori S, Tamura K. T lymphocyte activation and restenosis after percutaneous transluminal coronary angioplasty. *J Interferon Cytokine Res* 2001;21:219-221.

10. Andersen HO, Hansen BF, Holm P, Stender S, Nordestgaard BG. Effect of cyclosporine on arterial balloon injury lesions in cholesterol-clamped rabbits: T lymphocyte-mediated immune responses not involved in balloon injury-induced neointimal proliferation. *Arterioscler Thromb Vasc Biol* 1999;19:1687-1694.